FGF8 induces epithelial-mesenchymal transition and promotes metastasis in oral squamous cell carcinoma

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Abstract

Background Oral squamous cell carcinoma (OSCC) is one of the most common cancers worldwide, and with 500,000 new cases each year. The high risk of lymph node metastasis and local invasion are the main causes to cripples and death of OSCC patients. As potent growth factors, fibroblast growth factors (FGFs) not only exert biological effects for primary epithelial cells, but also make FGF signaling susceptible to being hijacked by cancer cells. However, the precise role of FGF8 and the therapeutic effects of FGF8 in OSCC need to be further investigated.

Methods Immunohistochemical staining was performed using in human OSCC tissues. Bioinformatics analysis was performed to analyze the potential FGF8-associated proteins. Migration and invasion of OSCC cells was examined by wounding healing assay and Matrigel assay. Expression of EMT related markers Was examined by immunoblot.

Results In this study, we show that FGF8 is upregulated in OSCC tissues and high FGF8 expression was related with a set of clinicopathologic parameters, including age, drinking, and survival time. FGF8 treatment enhances the invasive capability of OSCC cells. Lentivirus-based FGF8 expression promotes OSCC metastasis in a mouse lung metastasis model. Further, mechanistic study demonstrated that FGF8 induces epithelial-mesenchymal transition in OSCC cells.

Conclusions These results highlight a pro-metastatic role of FGF8, and underscore the role of FGF8 in OSCC development.

Background

Oral cancer ranks among the most frequent cancer worldwide, which is associated with severe morbidity and high mortality. Over 90% of oral cancers are oral squamous cell carcinomas (OSCCs)[1]. Many advanced therapeutic strategies applied in OSCC treatment[2, 3], however, the overall prognosis and the 5-year survival rate of OSCC patients are still less than 50%[4, 5]. The high risk of lymph node metastasis and local invasion are the main causes to cripples and death of OSCC patients. OSCC metastasis is a multiple and complex process; however, the key oncogenic factors are not fully illustrated[6]. Therefore, a better understanding of the mechanisms underlying OSCC metastasis is still needed.

There are 18 mammalian fibroblast growth factors (FGFs), which can be subdivided into 6 subfamilies based on protein sequence homology and phylogeny[7, 8]. FGF can act as morphogens, mitogens, and inducers of angiogenesis, when FGF bind and activate FGF receptors (FGFR), leading to activation of a series of biological processes [9–14]. FGFs are frequently upregulated in invasive tumors, making FGF signaling susceptible to be hijacked by cancer cells, facilitating tumor metastasis[7, 9, 10, 14]. It was reported that FGF1, FGF7 and FGF10 can induce EMT in bladder carcinoma cells[9]. FGF1, FGF2, FGF6, FGF9 and FGF17 have been showed to overexpress in prostate cancer[9, 10, 15, 16]. FGF8, FGF9, FGF10, FGF18, FGF23 were involved in the progression of colorectal cancer, and FGF9 expression negatively correlated with patients' survival[10, 12, 17, 18].

FGF8 is expressed in oral and maxillofacial tissues during embryonic development, and regulates epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) to facilitate organ formation. FGF8 expression disorder can lead to a variety of oral and maxillofacial developmental defect. In adults, FGF8 was reported that associated with diverse physiologic processes, including angiogenesis, wound repairing, homeostasis, cell differentiation and cell migration[19]. By contrast, FGF8 is rarely detected in normal adult tissues. However, aberrantly increased FGF8 expression is involved in the development of several forms of hormone cancers and engineered over-expression of FGF8 was found to promote invasion in animal models[12, 14, 20]. FGF8 can enhance the invasiveness and migration of prostate cancer cells and promote bone metastasis[20–22]. In a previous study, we reported that LRP6 promotes the expression of FGF8 in OSCC cells, and activation of LRP6 contributed to metastasis and poor prognosis in patients with OSCC. More importantly, in contrast to LRP6 expression alone, the concurrent expression of LRP6 and FGF8 could act as a better factor to predict OSCC patient prognosis[6]. However, the function of FGF8 alone in OSCC metastasis remains unclear.

In this study, FGF8 was found to be highly expressed in OSCC tissues, and was linked with an index of histopathological parameters. Further, we demonstrated that FGF8 treatment promotes EMT and induces an invasive phenotype in OSCC cells.

Methods

1. Clinical samples.

30 OSCC specimens containing adjacent noncancerous areas and 28 normal oral mucous tissues for immunohistochemical (IHC) analysis were collected from the Department of Oral and Maxillofacial Surgery, Hospital of Stomatology, Sichuan University. Demographic data and other variables, including dates of diagnoses, site and size of primary tumor, local regional recurrence, and distant metastasis were retrieved from the database provided by the oncology registry. The cancerous or noncancerous areas were identified by two pathologists independently, according to the IHC staining. The pathologists were blinded to patient clinical information. If the evaluations were controversial, the samples were re-evaluated and classified based on the assessment given most frequently by the pathologists. All the samples were obtained with patient's informed consent. The protocol of the study was approved by the Institutional Ethics Committee of West China Center, Sichuan University, China.

2. Immunohistochemistry

Immunohistochemistry Anti-FGF8 rabbit monoclonal antibody (ab81384, 1:200) was purchased from Abcam (Cambridge, MA, USA). Immunohistochemistry was detected on a slide carrying 4-mm-thick tissue from paraffin-embedded tumor species. After baked in a 37°C oven overnight, all slides were dewaxed in xylene and then rehydrated in ascending series of ethanol. Antigen retrieval was conducted by citrate antigen retrieval solution in an autoclave for 5min. Three percent of hydrogen peroxide was

incubated for 15min and normal goat serum working fluid incubated for 15min at 37°C after washing for 5min twice. Then the sections were exposed to the primary antibodies at 4°C in the wet box for one night. The slide tissues were washed in PBS for 5min three times and incubated secondary antibody for 15min at 37°C. DAB chromogenic reagents were used to detect the reaction of antigen and antibody and the slides were counterstained in hematoxylin, dehydrated in gradient alcohol, cleared in xylene.

To estimate the score of each section, eight individual fields were chosen by 2 dependent observers, and 100 cancer cells were counted for each field. We quantitatively scored the tissue sections according to the percentage of positively stained cells and staining intensity as described previously[23], with minor modifications. We assigned the following proportion scores: 0 if 0% of the tumor cells with positive staining, 1 if 0% to 10%, 2 if 11% to 30%, 3 if 31% to 70%, and 4 if 71% to 100%. We also rated the intensity of staining on a scale of 0 to 3: 0, negative; 1, weak; 2, moderate; 3, strong and 4, very strong. We then multiplied the proportion score by the intensity score to obtain a total score (range: 0-16). Scores were compared with overall survival duration, which was defined as the time from the date of diagnosis to death or the last known date of follow-up.

3. Bioinformatics analysis

Bioinformatics analysis of FGF8-associated proteins were performed following previous reports[24]. The protein-protein interaction (PPI) network was conducted based on the identified proteins, and biological evidence was collected from PrePPI to obtain the correlation of protein localization, the correlation of expression, the mutual binding, the upstream and downstream related proteins. Identified FGF8-associated proteins were classified according to the GO (Gene Ontology) Annotation clustering. The network group analysis was conducted via DAVID database (<http://david.abcc.ncifcrf.gov/>)[25].

4. Cell culture.

The HSC-3 and HSC-4 cell lines were provided by State Key Laboratory of Oral Diseases & National Clinical Research Center for Oral Diseases, West China Hospital of Stomatology, Sichuan University. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (Hyclone, USA), penicillin (10^7 U/L) and streptomycin (10 mg/L) at 37°C in a humidified chamber containing 5% CO₂.

5. Q-PCR

Total RNA of OSCC cell lines was isolated by TRIzol reagent (Invitrogen) and reverse transcript to cDNA with 1µg RNA in a volume of 20 µl by ExScript TM reagent kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The primers detailed sequences were as follows: FGF8: Forward Primer: 5'-CGC AAA GCT CAT TGT GGA GA-3', Reverse Primer: 5' ACA CGC AGT CCT TGC CTT TG-3'; GAPDH: Forward Primer: 5'-GAG TCA ACG GAT TTG GTC GT-3', Reverse Primer: 5'-TTG ATT TTG GAG GGA TCT CG-3'. Gene expression level was assessed by SYBR green qPCR SuperMix (Applied Biosystems Life Technologies, Foster, CA) and GAPDH served as an internal reference. The fold-change in the expression

of each target mRNA relative to GAPDH was calculated using the CT ($2^{-\Delta\Delta CT}$) method. Each experiment was conducted in triplicate.

6. Wound healing assay

When the cells cultured in 6-well plates reached approximately 100%, the wells were gently scratched with a 100 μ L pipette tip to create a uniform linear scratch. Then the cells were cultured in serum-free culture medium, and observed and photographed at 0h, 12h, and 36h. Cell migration was assessed by percent of wound closure through using Image-Pro Plus Analysis software (Media Cybernetics company, Rockville, MD). All experiments were conducted for three times to obtain the average value.

7. Transwell invasion assay

Invasion assays were carried out using 24-well culture plates containing the transwell chamber covered with Matrigel (1:4, BD, USA). 1×10^5 HSC-3 and 2×10^5 HSC-4 cells suspended in serum-free medium were placed in the upper chamber. 500 μ L medium containing 10% FBS were placed in the lower chamber. Cells remaining on the upper chamber were removed using a cotton swab after being incubated at 37°C for 12-36h, while cells traversed to reverse face of the membrane were fixed in 4% paraformaldehyde, stained with 1% Crystal Violet, washed three times with PBS, then air dried. The chamber was inverted on a microslide and observed under a microscope. Five fields per chamber were randomly selected for counting the number of invasive cells, and images were taken. Each experiment was conducted for three times.

8. In vivo tumor metastasis

All animals were humanely treated under the guidelines of the Institutional Animal Care and Treatment Committee of Sichuan University. 5×10^6 OSCC-FGF8 or OSCC-mock cells were injected into female athymic nude mice (10 mice per group) through the tail vein. Animals were sacrificed 28 days after injection. The lungs were excised and fixed in formalin for standard hematoxylin and eosin (H&E) staining.

9. Western blotting

After FGF8 treatment, total proteins of HSC-3 cells were extracted in RIPA buffer (50 mM Tris base, 1.0 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 1% cocktail) and quantified by coomassie brilliant G-250 (Bio-Rad). Samples were separated on 12% or 15% SDS- PAGE and then transferred to PVDF membranes. The membranes were blocked with 5% skim milk in TBST for 1h at 37°C and probed with primary antibody overnight at 4°C. After washing with TBST membranes were incubated with secondary antibody (1:5000 dilution; Santa Cruz Biotechnology) conjugated to horseradish peroxidase for 1h at 37°C. Finally, the proteins were detected by electro-chemiluminescence (ECL) Western blotting reagents. The following primary antibodies were used according to the manufacturer's instructions: anti-E cadherin mouse monoclonal antibody (ab1416, 1:1000), anti-Vimentin

rabbit polyclonal antibody (ab137321, 1:1000), Anti-Snail rabbit polyclonal antibody (ab82846, 1:800), and anti-GAPDH (ab8245, 1:1000, Abcam).

10. Immunofluorescence staining

The OSCC cells were cultured in 24-well cell culture plates, fixed for 15min with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 for 20 min. After blocking with normal goat serum working fluid for 1h at 37°C, primary antibody was incubated overnight at 4°C, and then staining was detected with fluorescein-conjugated secondary antibodies (PeproTech; 1:200) for 1h in dark condition. Finally, cells were stained with 4,6-diamidino-2-phenylindole (DAPI; blue) to show the nuclear position for 5min. Immunofluorescence signals were examined using a fluorescence microscope (Leica, Bensheim, Germany).

11. Lentiviral transduction

Expression of FGF8 were established using a pCDH Lentivector Expression System (System Biosciences, Mountain View, CA) according to the manufacturer's instructions. Briefly, the indicated shRNAs or cDNAs were cloned into pCDH lentiviral vector. Lentiviruses were produced by co-transfecting 293T cells with one of the expression plasmids and three packaging plasmids (pLP1, pLP2, pLP/VSVG). Infectious lentiviruses were harvested 72 h after transfection, centrifuged to remove cell debris, and filtered through 0.45 µm filter (Millipore, Bedford, MA).

Results

1. FGF8 is overexpressed in OSCC

To investigate the potential clinical role of FGF8 in OSCC, immunohistochemistry staining was performed on a panel of 30 OSCC specimens and 28 adjacent normal oral mucosa specimens. As shown in Figure 1a, FGF8 signal was positively detected in both the cytoplasm and membrane, whereas only weak staining of FGF8 was observed in majority of normal tissues (t-test; OSCC N=30, normal N=28; $P \leq 0.0001$; Figure 1b).

Next, we have evaluated the relevance between FGF8 expression and a series of clinicopathologic factors of OSCC patients. FGF8 immunoreactivity was more intense in tumor of elderly patients (one-way ANOVA; ≤ 40 N=3, 40-60 N=11, > 60 N=16; $P=0.0467$; Figure 1c). Further, the level of FGF8 expression was positively associated with drinking (t-test; with smoking N=14, without smoking N=16; $P=0.0014$; Figure 1d).

In a univariate analysis examining clinic-pathologic prognostic variables, the expression of FGF8 was significantly correlated with overall survival. A Kaplan-Meier survival analysis showed that subjects with high FGF8 expression had a significantly shorter 5-year overall survival time compared to those subjects with low FGF8 expression (log-rank test, $P=0.00282$, Figure 1e). These results showed that FGF8 is highly expressed in OSCC and may act as a potential prognostic marker for predicting patient outcome.

2. Analyses of FGF8-associated proteins

To explore the tumor-related function of FGF8, bioinformatic analyses was perform to screen the FGF8-related proteins (figure 2a). As a result, a total of 158 related proteins was extracted from Pre-PPI network and identified as FGF8-associated proteins. Next, we used the protein-functional GO annotation in the Kyoto encyclopedia of genes and genomes (KEGG) database to perform functional classification and signal pathway analysis of the associated proteins (figure 2b). Notably, two clusters of proteins, functioning in cell adhesion or migration, respectively, are found (figure 2c and d). These results suggested that FGF8 was likely involved in regulating OSCC metastasis.

3. FGF8 promotes OSCC tumor invasion and migration.

It has been demonstrated that FGF8 is involved in regulating migration and invasiveness in cancer cells[10, 12, 17, 18]. As a pilot test, FGF8 expressions in one normal oral squamous cell line (NOK) and four human OSCC cell lines (HSC-4, HSC-3, Cal-27, UM2) were examined. As shown in Figures 3a, FGF8 was less expressed in HSC-3 and HSC-4 cell lines at both RNA levels and protein levels. Therefore, HSC-3 and HSC-4 cell lines were selected as *in vitro* cell models.

The migratory and invasive capacities of OSCC cells were compared under FGF8 treatment at different concentrations. As shown in Figure 3b, FGF8 treatment promoted HSC-3 cells migration and increase the invasion potential, as demonstrated by wound healing assay and matrigel invasion. Similar results were observed in HSC-4 cells, therefore, such pro-migration and pro-invasive effect of FGF8 is not cell line-specific. These results demonstrate that FGF8 promotes migration and invasiveness in a dose dependent manner in OSCC cells.

4. FGF8 increases OSCC tumor metastasis in mice

To study the effect of FGF8 on tumor metastasis *in vivo*, FGF8 expression was induced in HSC-3 cells by a lentivirus-based system. FGF-8- or mock vector-expressed HSC-3 cells were intravenously injected into the nude mice to establish lung metastasis. The average number of metastatic nodules derived from FGF-8-expressed HSC-3 cells was 2.2-fold greater than control cells ($P < 0.05$; Figure 4a). In addition, the lung metastases areas formed by FGF-8-expressed HSC-3 was markedly larger than that formed by control cells, as determined by H&E staining (Figure 4b). These results showed that FGF8 has a positive impact on the OSCC cell metastases in mice model.

5. FGF8 promotes EMT in OSCC cells

EMT was considered among the initial steps during cancer metastasis[22]. Therefore, it was particularly interest to examine whether FGF8 plays a role in regulating OSCC cell EMT. As shown in Figure 5a, FGF8 treatment induced morphological changes in OSCC cells. HSC-3 and HSC-4 cells, which are both sub-rotund or sub-rectangular, changed into a spindle-like shape.

Furthermore, FGF8 treatment also reduced expression of the epithelial marker E-cadherin and increased the levels of mesenchymal markers Vimentin and Snail in HSC-3 and HSC-4 cells. These results suggested FGF8 induces a malignant phenotype by promoting EMT in OSCC cells in a time-dependent manner (Figure 5b and c).

Discussion

OSCC is one of the most common cancers world-wide, and the 5-year overall survival rate remains around 50%[26]. Although systemic therapeutic strategies, including chemo- and radiotherapy, have been developed, oral-facial disfigurement and functional defects in chewing, speaking, and swallowing are the major challenge in OSCC treatment [6, 27–29]. OSCC possesses poor prognosis since it is associated with a high risk of neck lymph node metastasis and uncontrolled local adjacent tissue invasion. Therefore, understanding the molecular mechanisms of OSCC metastasis is needed for improving OSCC treatment.

Epithelial-mesenchymal transition (EMT) is identified as part of the process of invasion and metastasis[30]. EMT can be characterized by changes in cell shape, in which epithelial cells become detached from each other, penetrate the basilar membrane and transform mesenchymal-like cells with a more flexible and migratory phenotype [31]. EMT can be induced by a variety of growth factors, including FGFs. FGFRs are activated after binding to cognate FGFs, and in turn trigger intracellular downstream signaling cascades via phosphorylating the tyrosine residue in their substrates. Increasing evidence indicates that aberrant FGF signaling are frequently observed in various tumors. Aberrant FGF signaling can function through modulating tumor–stroma interactions, or in a cell autonomous style, and activate different downstream pathways depending on cellular contexts. It is reported that FGF8 was involved in the regulation of colorectal cancer growth and metastasis by activating YAP1[12]. Our results showed a pivotal role of FGF8 in EMT in OSCC cell lines. In this context, the downregulation of epithelial markers, E-cadherin, and the upregulation of mesenchymal marker, vimentin and snail were detected. FGF8 regulates OSCC metastasis probably through inducing EMT. Further work is still needed to identify the intracellular effector proteins that promote EMT under FGF8 treatment.

Upregulation of FGF8 expression in OSCC tissues compared to normal tissues was revealed by immunostaining using clinical samples. The results also showed that FGF8 expression was strongly associated with the habit of drinking. Drinking is considered as one of important risk factors during tumor development. As a solvent, alcohol promotes the absorption of carcinogenic compounds on the mucosa surface, and damages the cell membrane and alters cell molecular composition. Long-term alcohol abuse

can cause salivary gland atrophy and lesions, saliva function and flow are affected. Therefore, the mucosal surface is locally exposed to a higher concentration of carcinogens to increase the risk of OSCC[32–35]. Notably, our results also showed that expression of FGF8 was negatively correlated with the survival time of patients, suggesting that FGF8 may be a potential indicator for OSCC prognosis.

Conclusions

In summary, our studies have provided evidences regarding the pro-metastatic role of FGF8 in OSCC cells. We also demonstrate aberrant upregulation FGF8 in OSCC, which is associated with the habit of drinking, and patient survival time. This study provides insight into the role of FGF8 in OSCC development, and contribute to improvement of OSCC treatment.

Declarations

Availability of data and materials

We declare that the data and materials in this study are provided free of charge to scientists for non-commercial purposes.

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Authors' contributions

RL contributed to the conception of the study. YH wrote the manuscript. All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

Ethical approval was provided by the Institutional Ethics Committee of West China Center of Sichuan University, China.

Consent for publication

All authors consent to publication.

Competing interests

The authors declare that they have no competing interests in this work.

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Figures

Figure 1

FGF8 is overexpressed in OSCC. (a) Representative images of FGF8 immunostaining using OSCC tissues and normal oral mucous tissues. (b) FGF8 immunostaining intensity in OSCC tissues and normal oral mucous tissues were analyzed. (c) FGF8 immunostaining intensity in OSCC patients among the different age groups were analyzed. (d) FGF8 immunostaining intensity in OSCC patients with or without drinking were analyzed. (e) Overall survival time of OSCC patients with high or low FGF8 expression was analyzed by Kaplan–Meier analysis.

Figure 2

Analyses of FGF8-associated proteins (a) The general workflow of bioinformatics analysis. (b) Sub-PPI network for FGF8-associated proteins, the predicted FGF8-associated proteins were divided into several groups based on their function. (c) The predicted FGF8-associated proteins involved in cell adhesion. (d) The predicted FGF8-associated proteins involved in cell migration.

Figure 3

FGF8 promotes OSCC tumor invasion and migration. (a) Expression of FGF8 in normal oral squamous cell line and several OSCC cell lines was examined by qRT-PCR and immunoblot. (b) HSC-3 or HSC-4 cells were incubated with active FGF8 recombinant protein at indicated concentrations for cell migration was examined by wound healing assay (upper two panels) and cell invasion was examined by Matrigel assay (bottom two panels). **, $P < 0.01$; ***, $P < 0.001$; ****,

Figure 4

FGF8 increases OSCC tumor metastasis in mice. (a) Representative images of lung metastasis and number of metastases following tail-vein injection with FGF8 or mock vector-expressed HSC-3 cells. *, $P < 0.05$. (b) H&E staining of the lung tissues.

Figure 5

FGF8 promotes EMT in OSCC cells (a) Representative phase-contrast images of cell morphology of HSC-3 and HSC-4 cells. (b and c) Expression of Snail, E-cadherin, and vimentin was examined by immunoblot (b) and immunofluorescent staining (c).